IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jonathan W. NYCE

Appl. No.: 09/543,679

Filed: April 4, 2000

For: **LOW ADENOSINE ANTI-SENSE OLIGONUCLEOTIDE, COMPOSITIONS, KIT & METHOD FOR TREATMENT OF AIRWAY DISORDERS WITH BRONCHOCONSTRICTION, LUNG INFLAMMATION, ALLERGY(IES) & SURFACTANT DEPLETION**

Art Unit: 1635

Examiner: Epps-Ford, Janet L.

Confirmation No. 6742

Atty. Docket: EPI-0067191 (02486.0025.CPUS01)

DECLARATION PURSUANT TO 37 CFR §1.132

Commissioner for Patents
Alexandria, VA 22313-1450

Sir/Madam:

I, Cynthia B. Robinson, M.D., do hereby declare as follows:

1. I am Vice President of Clinical Development at EpiGenesis Pharmaceuticals, Inc. (New Jersey, NJ, USA). I received my M.D. from Jefferson Medical College (Philadelphia, PA, USA) in 1982. I have over six years, since July 1997, of experience in the field of allergy immunology and pulmonary critical care; both of which encompass the treatment of asthma and other pulmonary diseases and disorders. My Curriculum Vitae is attached as Appendix A.

2. I am familiar with the prosecution history of the above-identified patent application and the patent application Ser. Nos. 09/093,972, filed June 9, 1998, and 09/016,464, filed January 30, 1998. I am submitting this declaration to show that antisense oligonucleotides that are complementary to genes other than an adenosine receptor are also effective in reducing expression of these genes thereby treating a pulmonary disease.

3. Experimental results are herewith provided to show that antisense oligonucleotides that are complementary to (1) bradykinin B2 receptor, (2) eotaxin, and (3) IL-4 receptor and IL-9 receptor are effective in reducing expression of these genes for treating a pulmonary disease. These experiments were performed under the direction of EpiGenesis Pharmaceuticals, Inc. The present application teaches using antisense oligonucleotides that targets one or more pulmonary and inflammation targets. The present application teaches a list of 160 such targets (pages 9-10, Table 1). Among these targets are: bradykinin B2 receptor (page 11, line 8), eotaxin (page 9, line 32), IL-4 receptor (page 9, line 30), and IL-9 receptor (page 9, line 38).

4. For the preclinical and clinical experiments, where the antisense oligonucleotides are administered as an aerosol, the PARI LC PLUS® Reusable Nebulizer (PARI GmbH, Starnberg, Germany) is used to generate the small particles for administering the antisense oligonucleotide formulation. The use of this nebulizer consistently produces aerosol particles of within the respirable range from 1 μm to 5 μm. The PARI LC PLUS® Reusable Nebulizer generates aerosol particles of a range of particle size of 1-5 μm (Coates, A.L., et al., *Chest* 113:951-6 (1998) and Todisco, T., et al., *J. Aerosol Med.* 8:97 (1995)). A study of seventeen different commercially available nebulizers showed that that the PARI LC PLUS® had a percent output in respirable range of $p<0.05$, and a respirable particle delivery rate of 0.24 ml/minute; both of which were significantly higher than the other models (Loffert, D.H., et al., *Chest* 106:1788-92 (1994)). .

5. **Oligonucleotide antisense to the bradykinin B2 receptor gene reduces expression of the bradykinin B2 receptor gene in an allergic rabbit model: DNA antisense therapy for asthma in an asthmatic rabbit model (administered in particle sizes of less than 5 μm).**

The experiments reported by Jonathan W. Nyce and W. James Metzger ("DNA antisense therapy for asthma in an animal model", *Nature* 385:721-5 (1997); attached as Appendix B), demonstrate that a 21-mer oligonucleotide antisense to the bradykinin B2 receptor gene, when

administered to an allergic rabbit model by aerosol, selectively attenuated bradykinin B2 receptor gene expression and resulted in reduced bradykinin B2 receptor number in airway smooth muscle in a dose-dependent manner (see the results in page 723, Figure 3 and page 724, Table 1). Table 1 in the Nyce and Metzger article corresponds to Table 5 of Example 20 (pages 310-311) of the present application. The aerosolized antisense oligonucleotides were generated by an ultrasound nebulizer that produced particles of less than 5 μm in diameter (see page 725, left column, lines 7-10). These results clearly demonstrate the gene-specific antisense effect of aerosolized oligonucleotides (antisense to the bradykinin B2 receptor gene) when administered in particle sizes of less than 5 μm to the lung of an asthmatic rabbit model.

6. <u>Oligonucleotides antisense to the eotaxin gene reduce expression of the eotaxin gene in an asthma mouse model: Antisense-Mediated Inhibition of Eotaxin Expression and Airway Eosinophilic Inflammation (administered in particle sizes of 10-50 μm).</u>

Eotaxin is an eosinophil-specific chemokine which recruits eosinophils to the site of allergic inflammation via binding to its receptor CCR3. Eotaxin is primarily secreted by epithelial cells, macrophage, T-cells, and eosinophils. Human studies have revealed high eotaxin concentrations in the BAL in atopic asthmatics compared to normal controls. (Lamkhioued et al., J. Immunol. 159: 4593-4601 (1997)). Similarly eotaxin expression is reportedly increased within the peripheral airways of the lungs of asthmatic subjects suggesting that eotaxin contributes to small airway and peripheral lung inflammation in asthma (Taha et al., J. Allergy Clin. Immunol. 103:476-483 (1999)). Asthma modeling studies using mice have largely supported the importance of eotaxin in allergic inflammation. Eotaxin deficiency or eotaxin antibodies have been reported to inhibit allergen-induced eosinophil recruitment to the lung (Gonzalo et al., J. Clin. Invest. 98: 2332-45 (1996); Rothenberg et al., J. Exp. Med. 185: 785-790 (1997); Schuh et al.; Am. J. Physiol. Lung Cell. Mol. Physiol. 283: L198-204 (2002)). Similarly, eotaxin gene transfer or exogenously administered eotaxin have been shown to augment allergen-induced eosinophil recruitment to the lung (Mould et al., J. Clin. Invest. 99: 1064-1071 (1997); Mould et al., J. Immunol. 164: 2142-2150 (2000)). Two studies have demonstrated that eotaxin deficiency does not completely abolish allergen induced eosinophil recruitment to the lung, suggesting that

while eotaxin plays an important role in eosinophil chemotaxis, other factors are involved (Yang et al., Blood 92: 3912-3923 (1998); Tomkinson et al., Int. Arch. Allergy Immunol. 126: 119-125 (2001)).

This study was designed to develop an eotaxin-specific antisense oligonucleotide (ASON), and demonstrate its efficacy *in vitro* and *in vivo*. An ASON was originally identified by screening a random antisense library against human eotaxin gene sequence using the human lung epithelial cell line A549. This antisense inhibited eotaxin mRNA expression and secreted eotaxin protein levels, while a 4 base-pair mismatch control did not have any significant effect. Analogous antisense and mismatch sequences were tested *in vivo* in a murine model of allergic asthma. BALB/c mice sensitized to chicken egg ovalbumin (OVA) were administered PBS, eotaxin antisense or mismatch oligonucleotides intranasally one day before, the day of, and two days following a single intranasal OVA challenge. Antisense and mismatch were administered at two doses, 10 mg and 100 mg. Two days after challenge, bronchoalveolar lavage (BAL) was collected and differential cell counts performed. Mice administered the 100 mg dose of eotaxin antisense demonstrated less BAL eosinophils ($6.2 \pm 1.2 \times 10^4$/ml) compared to the allergen control mice ($15.0 \pm 2.2 \times 10^4$/ml eosinophils; $p < 0.05$) which were sensitized and challenged with OVA, but received PBS instead of antisense or mismatch. The 10 mg dose of eotaxin antisense and both doses of mismatch did not reduce BAL eosinophil numbers relative to the allergic control group. In summary, an ASON specific for human eotaxin reduced eotaxin message and protein *in vitro*, and an analogous ASON specific for mouse eotaxin reduced eosinophilic inflammation in a mouse model of asthma. These findings provide preliminary evidence supporting the use of respirable ASONs against eotaxin in the treatment of eosinophilic inflammation in asthma.

***In vitro* methods**

Antisense Library: Antisense oligonucleotides able to inhibit eotaxin expression were originally identified by screening a random 20-mer antisense library generated against human eotaxin gene sequence using the human lung epithelial cell line A549.

Culture Conditions: Confluent monolayers of A549 cells were either treated eotaxin-specific antisense or mismatch control (5 mg/ml) (ASONs), in the presence of lipofectin (10

mg/ml), a carrier lipid, for 4 h followed by a 4h (for mRNA expression) or 18 h (for protein expression) treatment with the complete medium containing 10 ng/ml of TNF-a. mRNA expression was determined by TaqMan using specific eotaxin primers and probe. The level of secreted eotaxin protein in the conditioned medium of the A549 cells either untransfected or transfected with specific or control ASONs was determined by ELISA.

***In vitro* results**

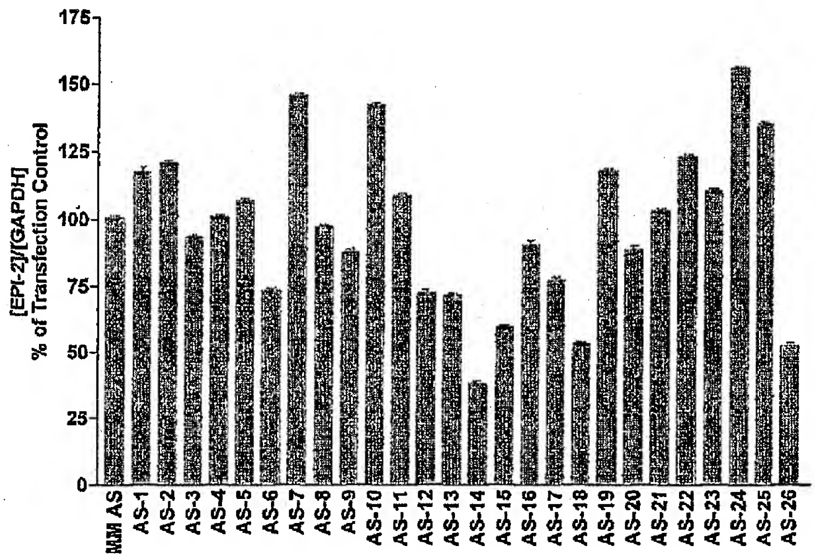

Figure 1. *In Vitro* Effect of Eotaxin AS on Eotaxin mRNA. TNF-a-stimulated A549 cells were treated with 5 mg/ml oligonucleotide in 10 mg/ml lipofectin for 4 hours and mRNA was collected after 4 additional hours. Results represent the means for triplicate samples

Table 1: Results of *In Vitro* Screen for AS-18

		Human Eotaxin mRNA (Percent of Control)
Human Eotaxin AS-18		**46%**
Human Eotaxin MM		**100%**

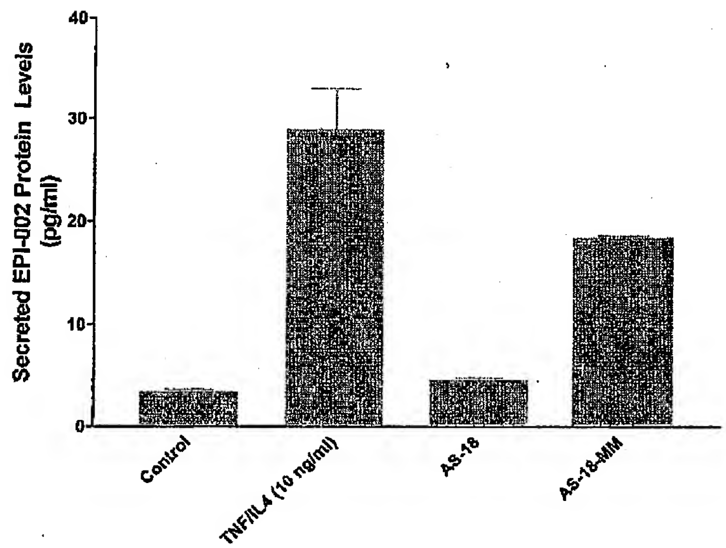

Figure 2. *In Vitro* Effect of Eotaxin AS on Eotaxin Protein. TNF-a-stimulated A549 cells were treated with 5 mg/ml antisense or mismatch oligonucleotide (AS-18 or AS-18-MM) in 10 mg/ml lipofectin for 4 hours and the cell supernatant was collected after an additional 18 hours. The mismatch oligonucleotide (AS-18-MM) was a 4 base pair mismatch relative to the antisense (AS-18). Results represent the means for triplicate samples

***In vivo* methods**

Mouse Antisense: An antisense sequence analogous to A-18 specific for mouse eotaxin, and free of CpG immunostimulatory sequences, was generated with a phosphorothioate backbone. A 4-base pair mismatch analogous to A-18-MM was also prepared.

Mouse Model of Allergic Asthma: 6-12 week old BALB/c mice were sensitized and challenged with 20 mg of chicken egg ovalbumin (OVA) allergen, and treated 3 times with saline, 10 or 100 mg eotaxin AS, or 10 or 100 mg of a four base mismatch control by intranasal insufflation (Figure 3). Intranasal insufflation involved administering particles (containing the eotaxin AS) that are 10-50 μm in size to the upper airways of the mice.

Bronchoalveolar Lavage (BAL): Mice were anesthetized, their tracheas cannulated, and the lungs were lavaged with 0.7 ml aliquots of ice cold PBS. BAL cells were pelleted, washed and counted using a hemocytometer, then plated on cytocentrifuge slides followed by

Wright/Giemsa staining. Differential counts of BAL eosinophils, neutrophils, lymphocytes, and macrophage/monocytes were performed by counting at least 250 cells per sample.

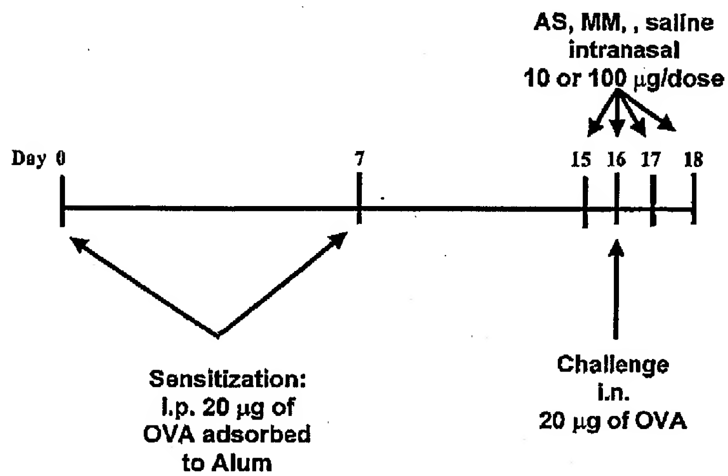

Figure 3. Mouse Sensitization, Challenge, and Treatment Protocol

In vivo results

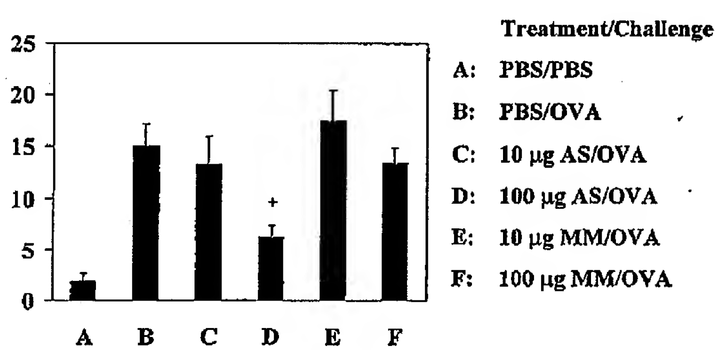

Figure 4. BAL eosinophilia

Total BAL eosinophils from allergen sensitized and challenged mice were counted following treatment with eotaxin AS, MM or vehicle control (PBS).

Conclusions

A library of 20-mer antisense oligonucleotides was developed based on the mRNA sequence of human eotaxin (Genebank accession code: NM_002986). Several antisense sequences produced substantial inhibition of eotaxin expression in an *in vitro* screening system using stimulated A549 cells. One of the inhibiting antisense, A-18, which contained no CpG immunostimulatory sequences, was shown to produce a 46% inhibition of human eotaxin expression and an 82% inhibition of secreted eotaxin protein. An analogous antisense sequence, specific for mouse eotaxin mRNA, was administered intranasally (in particles sizes of 10-50 μm) to sensitized and challenged mice in a murine model of allergic asthma, and shown to significantly inhibit allergen-induced BAL eosinophilia in a dose-dependent manner. Intrapulmonary antisense therapy directed against eotaxin appears to be a viable therapeutic modality for inhibiting allergen-induced eosinophil accumulation in the lung.

7. **Oligonucleotide antisense to a conserved region of the IL-4Ra and IL-9Ra genes reduces expression of these genes in an asthma mouse model: Simultaneous *In vivo* knockdown of IL-4Rα and IL-9Rα by a multi-target antisense oligonucleotide (administered in particle sizes of 10-50 μm).**

IL-4, IL-5, and IL-9 are pro-inflammatory cytokines involved in asthma pathogenesis. IL-4 is a key cytokine in development of the Th2 lymphocyte, which underlies the allergic phenotype, and stimulates immunoglobulin class switching in B-cells to produce IgE. L-5 promotes eosinophil growth, and longevity; and IL-9 stimulates mast cell growth and mucin production. Theoretically, an antisense oligonucleotide directed against a conserved sequence in receptors for IL-4, IL-5, and IL-9 could selectively suppress the expression of these receptors and inhibit the inflammatory process underlying asthma. The IL-4, IL-5, and IL-9 receptors (IL-4R, IL-5R, IL-9R) are heterodimeric complexes that link to JAK-STAT signaling pathways. IL-4R and IL-9R consist of unique cytokine-binding α subunits and a common IL-2$R\alpha_C$ subunit. IL-5R consists of a unique α subunit, and a β subunit that is common to IL-3 and GM-CSF. *In silico* analysis reveals a sequence of 16 bases that is conserved in the human IL-4Rα, IL-5Rα, and IL-9Rα and could be targeted by an antisense oligonucleotide. A 14 base segment of the human

sequence is also conserved in murine IL-4Rα and IL-9Rα, whereas the equivalent 14 base sequence in murine IL-5Rα exhibits two base mismatches.

A respirable antisense oligonucleotide (EPI-4067) targeting a conserved sequence in IL-4Rα and IL-9Rα was used to simultaneously inhibit two targets *in vivo*. Functionality against the separate targets was initially demonstrated *in vitro*. Incubating A549 cells with EPI-4067 and LipoFectin (cationic lipid) for 8 hours inhibited expression of IL-4Rα and IL-9Rα mRNA by 40% and 35%, respectively. In contrast, a mismatch (MM) control oligonucleotide had no effect. To demonstrate activity *in vivo*, Balb/c mice were sensitized by intraperitoneal injection of ragweed (RW) adsorbed to Alum, and challenged with RW intranasally. The mice were treated intranasally with 167 μg/day of EPI-4067 or MM for three consecutive days, starting one day before the RW challenge. Flow cytometry of single cells isolated from the lungs 24 hrs. after the last treatment revealed reduced expression of IL-4Rα and IL-9Rα, but not IL-5Rα, in animals treated with EPI-4067 compared to MM. Splenocytes exhibited no difference in expression. Expression in lungs cells returned to normal by 6 days after the last treatment. IgE production, which is under the control of IL-4 and IL-9, was inhibited by EPI-4067, as indicated by a significant reduction in serum total IgE 6 days after treatment. These results demonstrate that multiple targets can be selectively down regulated *in vivo* through administration of multi-target antisense oligonucleotides. Thereby demonstrating that an antisense oligonucleotide (EPI-4067) selectively inhibits the expression of IL-4Rα and IL-9Rα in a mouse model of asthma.

Methods

Antisense was screened for effects on IL-4Rα mRNA *in vitro* using PMA-activated A549 (human adenocarcinoma) cells. Cells were treated with 1 μM EPI-4067 in Lipofectin.

Animals were sensitized and challenged with 80 μg of ragweed (RW) allergen, and treated 3 times with saline, 167 mg EPI-4067 (5'-CTC-CAC-TCA-CTC-CA-3'), or 167 mg of a 4 base mismatch control (5'-CTC-ACT-CAC-TCC-CA-3') by intranasal insufflation (Figure 1). Intranasal insufflation involved administering particles (containing the EPI-4067 antisense oligonucleotide) that are 10-50 μm in size to the upper airways of the mice. Serum was collected by cardiac puncture and total IgE levels were measured by ELISA. Lungs were isolated, minced,

and digested with collagenase. Single cells were collected, and expression of IL-4R, IL-5R and IL-9R was measured cytometrically.

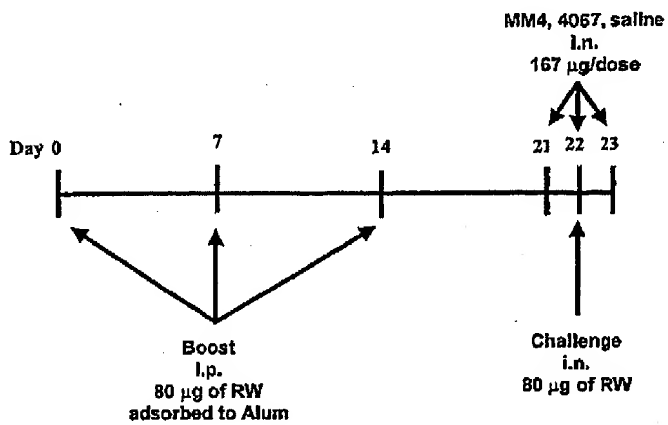

Figure 1. Sensitization, Challenge, and Treatment Protocol

Table 1. Results of Initial *In vitro* screen

		IL-4R mRNA (% of Control)
Wobble		100%
MM4		92%
EPI-4067		60%

PMA-stimulated A549 cells were treated with 1 mM oligonucleotide in Lipofectin Results represent the means for duplicate samples.

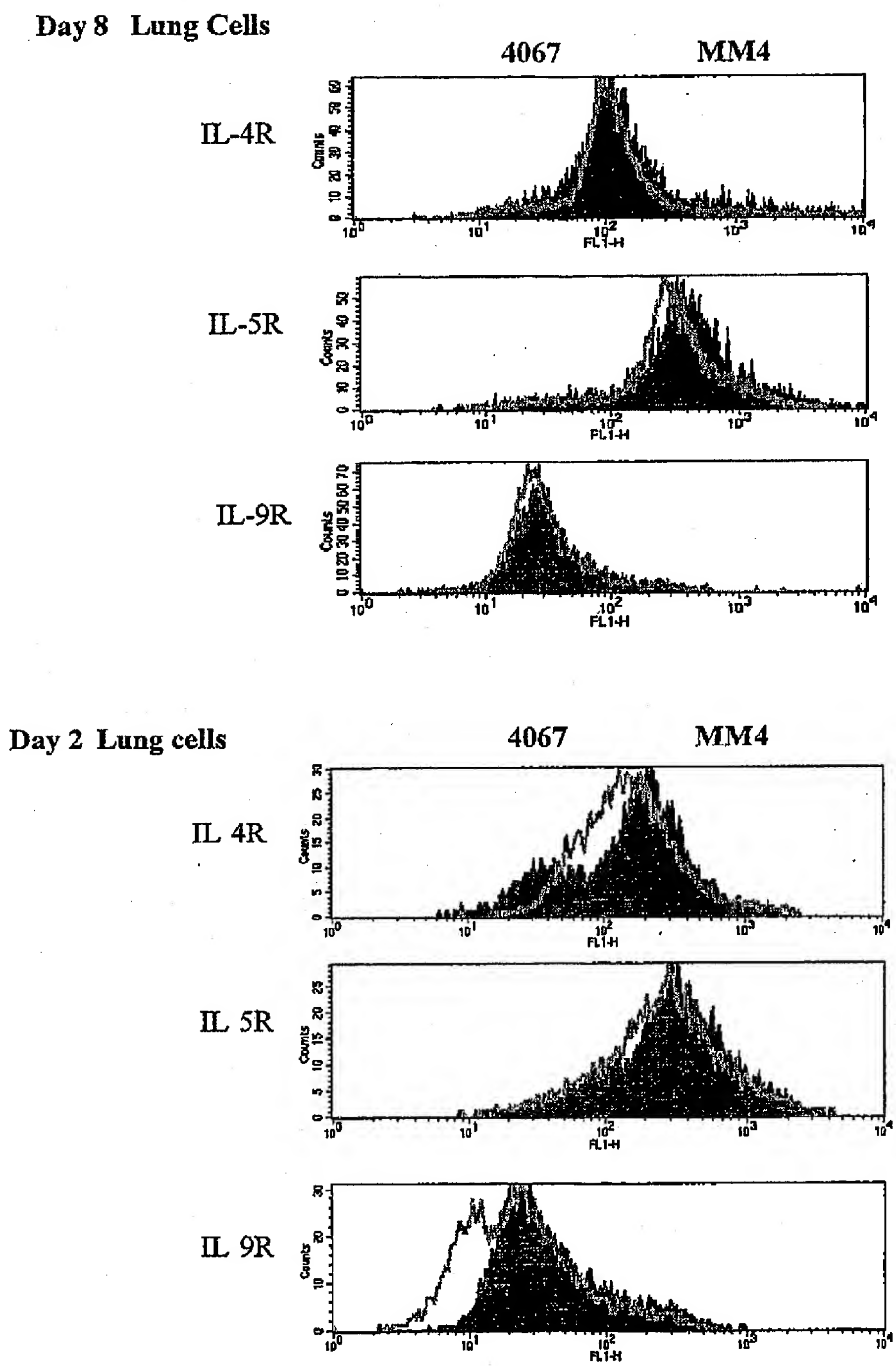

Figure 2. Expression of IL-4, IL-5 and IL-9 receptors in lung digests

Day 2 post-challenge, single cell preparations from lung digests showed inhibition of IL-4R, IL-5R and IL-9R expression by 4067 treatment compared to MM4 treatment. No difference in expression was observed in spleen cells (data not shown). Day 8 post-challenge, expression of IL-4R, IL-5R and IL-9R in lung cells was not different between the EPI-4067 and mismatch control groups.

Day 2 Spleen cells

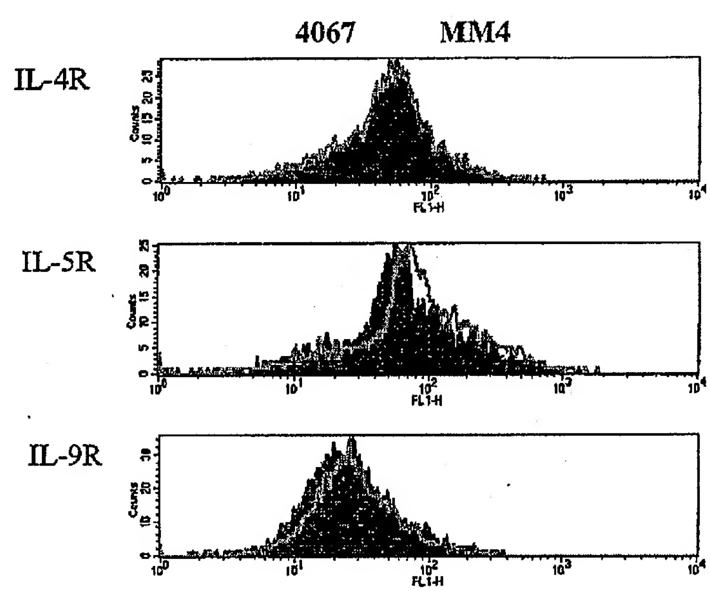

Figure 3. Effect of EPI-4067 on receptor expression in spleen cells

2 days after challenge, a time when IL-4R, IL-5R and IL-9R in lung cells was inhibited, there was no inhibition of receptor expression observed in the spleen

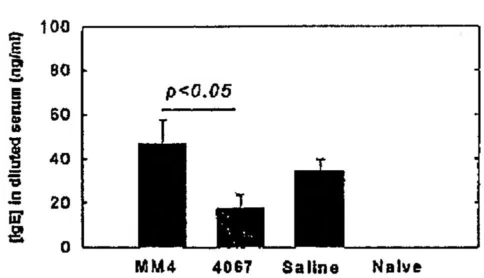

Figure 4. Serum total IgE concentration 2 days after challenge

Serum IgE levels in serum isolated 2 days after challenge were determined by ELISA. The group of mice that were treated with 4067 resulted in a significant decrease of total serum IgE compared to a group of MM4 treatment. Individual serum was diluted at 1:40 before assay (Saline n=3, Others n=4)

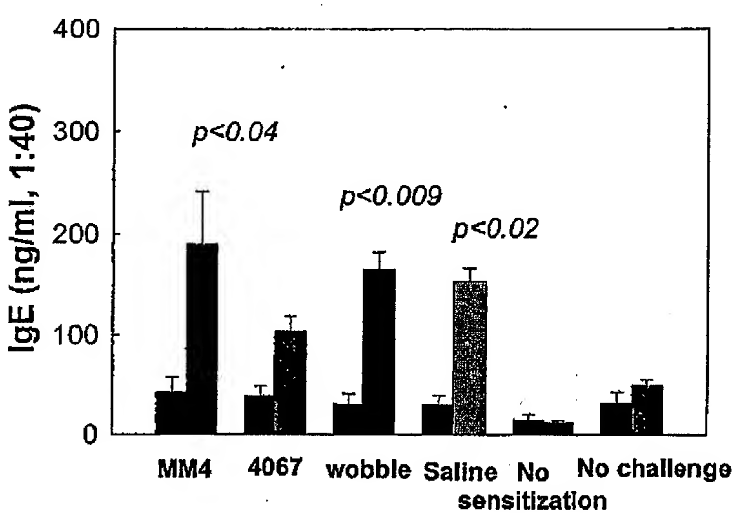

Figure 5. Serum total IgE concentration 6 days before and 6 days after challenge

IgE levels were determined in serum collected 6 days before (left columns) and 6 days after ragweed challenge (right columns). IgE was elevated in all groups that were sensitized and challenged. The group treated with EPI-4067 exhibited significantly less IgE compared to groups treated with mismatch, wobble, or saline. Individual serum was diluted at 1:40 before assay [n=15 except for No challenge (n=10) and MM4 (n=5)].

Conclusions

Respirable antisense targeting a conserved sequence in the IL-4Rα and IL-9Rα, when administered in particles sizes of 10-50 μm, is effective in reducing protein expression of both receptors in the lungs but does not alter expression in splenocytes. Expression of IL-5Rα, which exhibits a 2 base mismatch when compared to the conserved sequence in IL-4Rα and IL-9Rα, is not affected. IgE production, which is controlled by IL-4, is likewise inhibited.

8. Treatment of diseases by use of antisense oligonucleotides has the potential of being administered in a variety of means. Typically, such administration is in the form of injection, as this mode of administration has been used by investigators for treatment of diseases using antisense oligonucleotides. This mode of administration permits controlled administration of the drug and potential systemic treatment of a target disease. However, recent studies (subsequent to the effective filing date of the present invention) have shown that injection or oral administration of drugs to treat respiratory diseases such as asthma have not been effective (e.g., use of dehydroepiandrosterone for the treatment of asthma). Direct administration of drugs to the airways may be problematical in controlling the dosage and proper adsorption of the drug at the site. The results obtained using the present invention demonstrate the clear superiority of treating respiratory diseases by controlling the particle size of the drug and administering the drug directly to the airway of the patient over the systemic treatment. In my opinion, the present invention provided unexpectedly superior results of efficacy as compared to the results one would expect if the oral or injectable treatment were used. Specifically, by providing small particle size of 1-5 μm for small airway deposition or 0.5 μm to 500 μm for upper airway deposition one is able to achieve a higher concentration of the antisense oligonucleotide drug at the specific locality where it is to interact with its target polynucleotides. The patient is able to respire or inhale the drug in this small particle size into the airway or lungs via suitable delivery means such as a nebulizer, a dry powder inhaler, a propellant-driven system or by insufflation. Different methods of generating respirable particles produce particles capable of deposition in different parts of the airway. For example, insufflation produces larger particles (10-50 μm) that remain primarily in the upper airways.

9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the applications or any patent issuing thereon.

Respectfully submitted,

Dated: November 21, 2003

Cynthia B. Robinson, M.D.
Vice President of Clinical Development
EpiGenesis Pharmaceuticals, Inc.

Appendix A

CURRICULUM VITAE

CYNTHIA BROUSE ROBINSON
M.D.

CURRICULUM VITAE

CYNTHIA BROUSE ROBINSON, M.D.
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A DATE: 24-Nov-2003

B BIOGRAPHICAL INFORMATION

BIRTHPLACE:	Washington, DC	
CITIZENSHIP:	USA	
MARITAL STATUS:	Married, David M. Robinson, MD	
CHILDREN:	Kelly Christine	4/17/85
	Matthew Karl	12/17/88
PRESENT TITLE:	VP, Clinical Development, EpiGenesis Pharmaceuticals	

C CAREER OBJECTIVES

Long-term:

- Manage and provide strategic focus for a clinical group responsible for development of a balanced portfolio, including marketed products and early phase compounds, preferably in pulmonary. Direct multidisciplinary team to submit and gain approval of NDAs, sNDAs.

Near term:

- Direct and develop a multidisciplinary development team to progress compounds from target validation through to, including clinical proof-of-concept in respiratory disease. Acquire understanding of biotechnology business aspects and deliverables. Immersion into strategic business development to add value to the company. Expand experience in CMC, toxicology and regulatory affairs for pulmonary

products. Acquire additional experience in pivotal trial conduct. Acquire additional experience in deriving target product profiles and developing and implementing clinical development plans to realize key TPP elements and maximize their value. Participate in multidisciplinary team engaged in evaluating in-licensing opportunities.

D **INDUSTRY EXPERIENCE:**

1/02-current
VP, Clinical Development, EpiGenesis Pharmaceuticals
Responsible for all preclinical and clinical development of respiratory compounds including small molecules and inhaled oligonucleotide compounds (NME). Responsible for supervising regulatory affairs, CMC, quality assurance and clinical development. Responsible for supervision of CRO support including data management, biostatistics, manufacturing, packaging, toxicology, clinical study support. Together with VP, CSO jointly responsible for integration of discovery and development objectives. Supervision of 5 direct reports. Chief medical officer. Responsible for clinical development of EPI 2010 (phase II asthma), EPI 12323 (preclinical development) including TPP, development plans and clinical study design.

2/01-1/02
Director, Clinical Drug Discovery, Respiratory, Inflammation, Respiratory Pathogens, CEDD
Responsible for early clinical development (candidate selection through IIa) of pulmonary and rheumatoid arthritis compounds with a major focus on COPD including:

- development of TPP for RA and COPD indications
- development of asset product profiles for 9 early compounds in portfolio
- development of early clinical development plans
- development of clinical protocols to support early development
- recruit/train/supervise of physicians/scientists
- development of mechanism of action protocols using novel/surrogate endpoints in COPD and RA
- preparation of strategic disease area review documents
- preparation of regulatory reports
- maintenance of budgets/contracts for CEDD-sponsored studies

8/99-2/01
Director, Pulmonary/Diabetes Clinical Research and Medical Affairs SmithKline Beecham Pharmaceuticals
Responsible for clinical development (phase II and III) of pulmonary compounds including :

- development of clinical plans to fit TPP

- conduct of large phase III studies (study operations, budgeting, monitoring, medical oversight)
- preparation of reports and regulatory documents (oversight of data handling, data interpretation, IB, ISS)
- leadership of matrix functional teams including chair of clinical working groups
- medical affairs support by participation in publication review. Experience with commercial-clinical interface and KOL development.
- extensive experience with discovery-clinical interface by providing input to early development strategy including Go/No Go decision points, appropriate experimental models, extrapolation of animal data to patient treatment settings
- supervisory role to clinical research scientists
- Member of the following teams: 1) US Med Director IL-5 Mab, 2) Cilomilast (Ariflo) asthma program, 3) Cilomilast mechanism of action studies, 4) Cilomilast CR COPD program (shared responsibility) 5) p38 MAP kinase inhibitor program (pulmonary indications), IL-8 receptor antagonist (pulmonary indications)

7/97-8/99
Director, Clinical Pharmacology SmithKline Beecham Pharmaceuticals
Responsible for early clinical development (phase I) and experimental medicine of lead and back-up compounds in a variety of therapeutic areas including:

- preparation of early clinical development plans including experimental studies and proof-of-compound activity studies
- preparation of reports, (32) protocols (15) and regulatory documents (INDs, IND updates)
- conduct of phase I studies including first-into-man protocols
- extensive interface with IRB and instruction /education about good clinical practice
- provision of pulmonary expertise for in-licensing opportunities, due diligence provided on safety and efficacy for antihistamine compound
- supervision and medical director of Clinical Laboratory (two direct reports, 50 indirect reports)
- supervision and mentorship of Assist. Director, H. Chou, M D, PhD
- Responsible compounds: IL-4 Mab, NK3 receptor antagonists (lead and back-up compounds), Osteoclast vitronectin receptor antagonists, including development of experimental medicine model of accelerated bone resorption model (lead and back up), Ornade/Lithium spansules, endothelin receptor antagonists (IV and oral formulations) including MOA study.

E **EDUCATION:**

9/72 - 10/76	B.S.	Northwestern University, Evanston, IL (Physical Therapy)
9/78 - 6/82	M.D.	Jefferson Medical College, Philadelphia, PA

F **POSTGRADUATE TRAINING AND FELLOWSHIP APPOINTMENTS:**

7/82 - 6/85	Internship and Residency, Department of Internal Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA
7/85 - 6/88	Fellowship, Cardiovascular/Pulmonary Division, Department of Internal Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA
7/88 - 6/90	Fellowship, Division of Pulmonary and Critical Care Medicine, University of California, Davis, Medical Center, Sacramento, CA

G **FACULTY APPOINTMENTS:**

7/90 - 3/94	Assistant Professor in Residence, Division of Pulmonary and Critical Care Medicine, University of California, Davis, School of Medicine, Sacramento, CA
3/94 – 7/97	Assistant Professor of Medicine, Pulmonary and Critical Care Division, Department of Internal Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA
7/97- present	Assistant Adj. Professor of Medicine, Pulmonary and Critical Care Division, Department of Internal Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

H **HOSPITAL AND ADMINISTRATIVE APPOINTMENTS:**

3/94 - present	Clinical Director, Adult Cystic Fibrosis Program, Hospital of the University of Pennsylvania, Philadelphia, PA
3/94 – 7/97	Clinical Director, Human Gene Therapy Program, Hospital of the University of Pennsylvania,

	Philadelphia, PA
7/97 –8/99	Director, Clinical Pharmacology SmithKline Beecham Pharmaceuticals
8/99-present	Director, Pulmonary/Diabetes TU

I SPECIALTY CERTIFICATIONS:

1983	National Board of Medical Examiners
1985	American Board of Internal Medicine
1988	Subspecialty Certification in Pulmonary Medicine
1989	Subspecialty Certification in Critical Care Medicine
1999	Renewal Certification in Critical Care Medicine

J LICENSURE: California #G62920
Pennsylvania #030855-E-MD

K AWARDS, HONORS AND MEMBERSHIPS IN HONORARY SOCIETIES:

1981 Alpha Omega Alpha, Medical Honor Society

L MEMBERSHIPS IN PROFESSIONAL AND SCIENTIFIC SOCIETIES:

National Society:

American Thoracic Society

Local Society:

Pennsylvania Thoracic Society

Consultant to ARI INC., preclinical asthma formulation. 1997.

Consultant to Cortech, International for pseumomonas vaccine in CF. 1997.

M PRINCIPAL INVESTIGATOR OF GRANTS:

"Regulation of Fibronectin mRNA by TGFb." University of California, Davis — Young Investigator's Award, American Lung Association of California, $19,000. 7/1/90-6/30/91.

"Regulation of Fibronectin mRNA by TGFb." University of California, Davis — Francis B. Parker Fellowship Award, $96,000. 8/1/90-7/31/93.

"TGFb and TGFa Gene Expression by Cigarette Smoke." University of California, Davis — California Tobacco-Related Disease Research, $75,000/year. 7/1/90-6/30/93.

"The Role of Fibronectin in Tracheal Epithelial Cells." University of California, Davis — Young Investigator's Award, American Lung Association of California, $15,000. 7/1/91-6/30/92.

M **PRINCIPAL INVESTIGATOR OF GRANTS (CONT'D):**

"Safety and Efficacy of Aerosolized Adenovirus Containing CFTR in Mammals." University of Pennsylvania — Cystic Fibrosis Foundation $100,000. 8/1/93-7/30/95.

"Gene Therapy for Cystic Fibrosis Lung Disease Using Second Generation Adenovirus." Project 3 University of Pennsylvania — NIDDK, $1,085,720. 9/30/94-9/29/99.

"A Randomized, Double-Blind Multicenter Study Evaluating the Effect of Montelukast Sodium to Salmeterol on the Inhibition of Exercise-Induced Bronchoconstriction." Merck & Co $32,688. 10/01/96-10/01/97.

Epidemiologic Study of Cystic Fibrosis" Genentech, Inc. $42,000.00. 11/01/94-11/01/98.

"A Phase IV Multicenter Randomized Trial in Patients with Cystic Fibrosis to Determine the Relative Efficacy of Pulmozyme Delivered by two Different Systems." Genetech, Inc. $8,500. 12/01/95-12/01/96.

"Long Term Safety Study of Zileuton Controlled-release Plus Usual Care Versus Placebo Plus Usual Care in Patients with Asthma." Abbott Laboratories. 3/18/97-3/18/98.

N **MAJOR TEACHING RESPONSIBILITIES FOR THE UNIVERSITY OF PENNSYLVANIA:**

Outpatient chest clinic and adult CF program 4/ month
Administration of the adult CF program 4/ month

O **CHAPTERS:**

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Appendix B

Nyce, J.W., *et al.*, "DNA antisense therapy for asthma in an animal model", *Nature* 385:721-5 (1997)

DNA antisense therapy for asthma in an animal model

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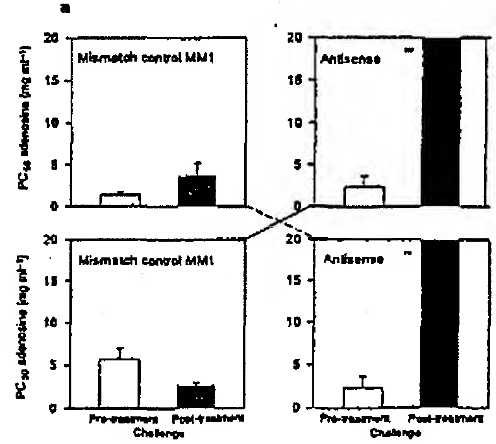
Asthma is an inflammatory disease characterized by bronchial hyper-responsiveness that can proceed to life-threatening airway obstruction. It is one of the most common diseases in industrialized countries, and in the United States accounts for about 1% of all healthcare costs[1]. Asthma prevalence and mortality have increased dramatically over the past decade[2], and occupational asthma is predicted to be the pre-eminent occupational lung disease in the next decade[3]. Increasing evidence suggests that adenosine, an endogenous purine that is involved in normal physiological processes, may be an important mediator of bronchial asthma[4–15]. In contrast to normal individuals, asthmatic individuals respond to adenosine challenge with marked airway obstruction[6,7], and concentrations of adenosine are elevated in the bronchoalveolar lavage fluid of asthma patients[9]. We performed a randomized crossover study using the dust mite-conditioned allergic rabbit model of human asthma. Administration of an aerosolized phosphorothioate antisense oligodeoxynucleotide targeting the adenosine A_1 receptor desensitized the animals to subsequent challenge with either adenosine or dust-mite allergen.

a



b

PC50 Adenosine					
A_1 MM Control		A_1 MM2 control		A_1AS	
Pre ODN	Post ODN	Pre ODN	Post ODN	Pre ODN	Post ODN
3.56 ± 1.02	3.25 ± 0.34	2.46 ± 0.50	2.81 ± 0.70	2.36 ± 0.68	>19.5 ±0.34**

Figure 1 a. Effects of adenosine A_1 receptor antisense ODN upon PC_{50} values in asthmatic rabbits. PC_{50} adenosine values were determined before and after intratracheal administration of aerosolized A_1AS or A_1MM to allergic rabbits. After a two-week rest period between parts of the experiment, rabbits were then crossed over, with those that had received A_1AS in the first part now receiving A_1MM, and those that had received A_1MM in the first part now receiving A_1AS. A_1MM2-treated animals were a separate group. b, Data summary. Results are presented as the mean ± s.e.m. Significance was determined by repeated-measures ANOVA and Tukey's protected *t*-test. Asterisks indicate a significant difference from all other groups, $P < 0.01$.

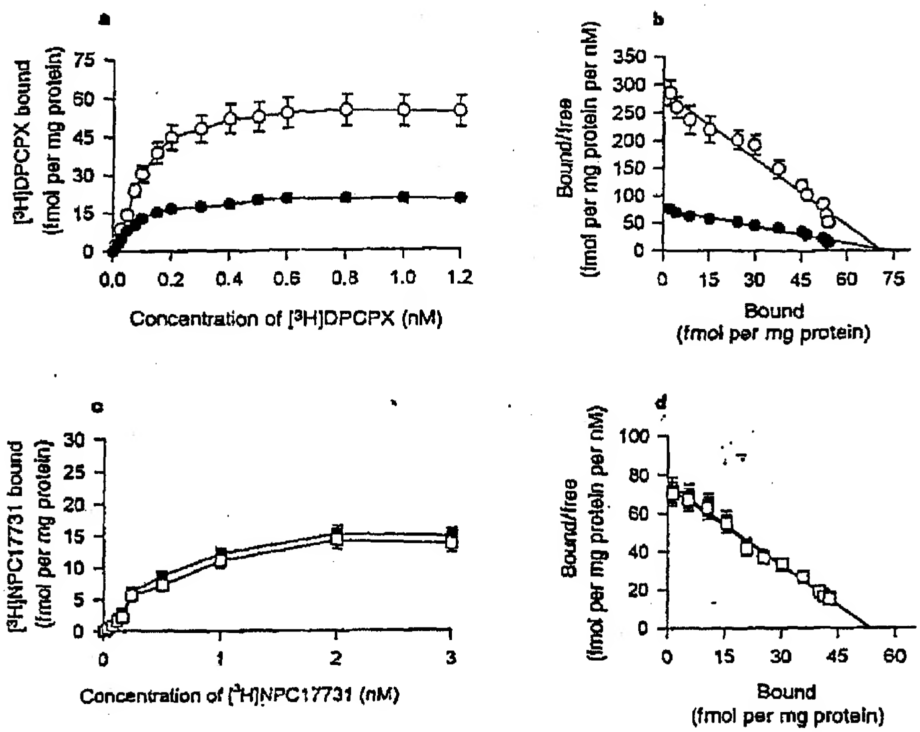


Figure 2 Specificity of action of adenosine A_1 receptor antisense ODN A_1AS. Airway smooth muscle tissue was dissected from rabbits administered a total of 20 mg A_1AS or A_1MM in four divided doses over 48 h. Plasma-membrane fractions were prepared. **a**, Saturation isotherm of [³H]DPCPX binding to allergic rabbit lung plasma membrane from A_1AS- (filled circles) and A_1MM-treated (open circles) allergic rabbits showing an approximate 75% decrease in adenosine A_1 receptor number in airway smooth muscle from A_1AS-treated animals. **b**, Scatchard plot of saturation isotherm from **a** indicating a single class of binding sites; A_1AS (filled circles), A_1MM (open circles). **c**, Saturation isotherm of [³H] NPC17731 binding to allergic rabbit lung plasma membrane from A_1AS- (open squares) and A_1MM-treated (filled squares) allergic rabbits showing no change in bradykinin B_2 receptor number in airway smooth muscle of A_1AS-treated animals. **d**, Scatchard plot of saturation isotherm from **c** indicating a single class of binding sites. A_1AS (open squares), A_1MM (filled squares). Error bars represent s.e.m.

Antisense oligodeoxynucleotides (ODNs) induce functional gene ablation by degenerating the template activity of specific target mRNAs[16,17]. We considered the lung to represent an excellent potential target for aerosolized antisense ODNs, for several reasons. The lung can be approached non-invasively and relatively specifically by inhaled aerosolized ODNs; it has a very large absorption surface ($150\,m^2$ in the human); and it is lined with surfactant, a material that could potentially facilitate the pulmonary distribution and intracellular uptake of respired ODNs. In this regard, cationic lipids have been used to enhance cellular uptake of antisense ODNs[18,19], and dipalmitoylphosphatidylcholine, a major constituent of surfactant, is a zwitterionic lipid that can act as a weak cation at physiological pH. Indeed, a surfactant-based delivery system for transfection of airway cells with DNA has been described[20]. Other aspects of the physiology of surfactant, for example its high rate of recycling between the alveolar surface and the pulmonary epithelium[21], might also potentially facilitate pulmonary distribution and uptake of respired ODNs. We considered bronchial hyperresponsiveness in the allergic rabbit model of human asthma to be an excellent endpoint for antisense application because the tissues involved in this response lie near the point of contact with aerosolized ODNs, and the model closely simulates an important human disease. Furthermore, a serendipitous homology between the human and rabbit adenosine A_1 receptors centring on the initiation codon allowed us to use in the allergic rabbit model an antisense ODN (A_1AS) designed to target the human adenosine A_1 receptor mRNA.

In the first part of the experiment, four randomly selected allergic rabbits were administered A_1AS, and four were administered a mismatched control, A_1MM. On the morning of the third day, PC_{50} values (the concentration of aerosolized adenosine required to reduce the dynamic compliance of the bronchial airway 50% from the baseline value) were obtained and compared with PC_{50} values obtained for these animals before exposure to ODN. The experiment was repeated two weeks later in crossover fashion, with the animals previously treated with A_1AS now receiving the mismatched control A_1MM, and the animals previously treated with A_1MM now receiving A_1AS. Another group of four animals was administered a second mismatch control, A_1MM2. The results of this experiment are shown in Fig. 1. In both parts of the experiment, animals receiving the antisense ODN showed an increase of at least an order of magnitude in the dose of aerosolized adenosine required to reduce dynamic compliance of the lung by 50%. No effect of the mismatched control ODNs upon PC_{50} values was observed. A_1AS desensitized allergic rabbits to adenosine in a dose-dependent fashion over a range of 0.2, 2.0 and 20.0 mg total dose, and A_1MM was without effect over this same dose range.

When the crossover experiment was completed, airway smooth muscle was surgically dissected from all of the rabbits and processed for quantitative assessment of adenosine A_1 receptors. As a control for specificity of the antisense ODN, adenosine A_2 receptors and bradykinin B_2 receptors were also quantified. Rabbits treated with A_1AS in the crossover experiment had a nearly 75% decrease in A_1

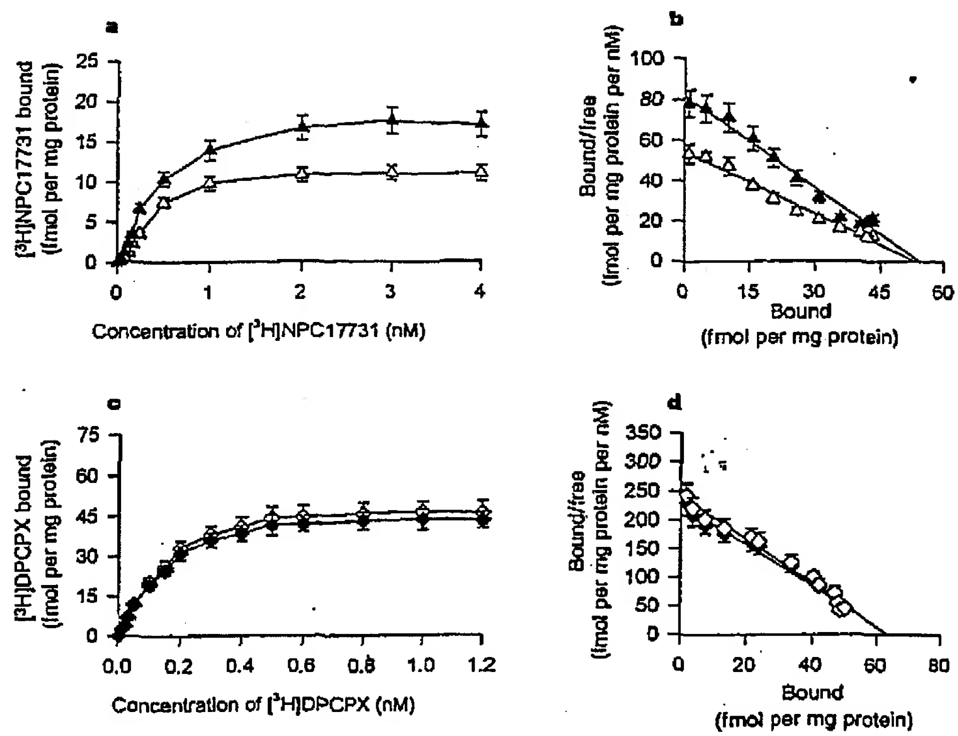


Figure 3 Specificity of action of bradykinin B_2 receptor antisense ODN B_2AS. Airway smooth muscle tissue was dissected from rabbits administered 20 mg B_2AS or B_2MM in four divided doses over 48 h. Plasma-membrane fractions were prepared. **a**, Saturation isotherm of [^{3}H]NPC17731 binding to allergic rabbit lung plasma membrane from B_2AS- (open triangles) and B_2MM-treated (filled triangles) allergic rabbits showing an approximate 40% decrease in bradykinin B_2 receptor number in airway smooth muscle from B_2AS-treated animals. **b**, Scatchard plot of saturation isotherm from **a** indicating a single class of binding sites; B_2AS (open triangles), B_2MM (filled triangles). **c**, Saturation isotherm of [^{3}H]DPCPX binding to allergic rabbit lung plasma membrane from B_2AS- (open diamonds) and B_2MM-treated (filled diamonds) allergic rabbits showing no change in adenosine A_1 receptor number. **d**, Scatchard plot of saturation isotherm from **c** indicating a single class of binding sites; B_2AS (open diamonds), B_2MM (filled diamonds).

receptor density compared with controls (Fig. 2), as assayed by specific binding of [^{3}H]-DPCPX. This effect occurred in a dose-dependent fashion over the range 0.2, 2.0 and 20.0 mg total dose. There was no change in adenosine A_2 receptor density, as assayed by specific binding of the A_2 receptor-specific ligand 2-[-p(2-carboxyethyl) - phenethylamino] - 5′- (N - ethylcarboxamido) adenosine (CGS-21680), or in bradykinin B_2 receptor density, as assayed by specific binding of the bradykinin B_2 receptor-specific ligand NPC17731, over this same dose range of A_1AS. Scatchard analysis of the binding isotherm of [^{3}H]-DPCPX to membranes prepared from bronchial smooth muscle isolated from allergic rabbits treated with 20 mg A_1AS yielded K_d and B_{max} values of 0.36 nM and 19 fmol mg^{-1} protein, respectively, compared with values of 0.34 nM and 52 fmol mg^{-1} protein, respectively, for rabbits treated with control A_1MM ODN (Fig. 2). This confirms that there is effective and selective attenuation by A_1AS of a single class of adenosine receptors of the A_1 type.

As a further control to demonstrate gene-specific effects in this model system, an antisense ODN targeting the bradykinin B_2 receptor (B_2AS) was administered as an aerosol to allergic rabbits under the same conditions as for A_1AS. Like adenosine, bradykinin is a potent bronchoconstrictor agent in asthmatic airways[22], and this effect is thought to be mediated through the B_2 receptor[23,24]. Aerosolized B_2AS specifically downregulated B_2 receptor binding by the B_2 receptor-specific ligand [^{3}H]-NPC17731 in airway smooth muscle of allergic rabbits (Fig. 3a, b). Neither adenosine A_1 nor A_2 receptor binding by their specific ligands was affected by B_2AS over the dose range 0.2, 2.0 and 20.0 mg. A minimally mismatched control molecule, B_2MM, was without effect on any receptor over this same dose range. Scatchard analysis of the binding isotherm of [H^3]-NPC17731 to membranes prepared from bronchial smooth muscle isolated from allergic rabbits treated with 20 mg B_2AS yielded K_d and B_{max} values of 0.38 nM and 8.7 fmol mg^{-1} protein, respectively, compared with values of 0.41 nM and 14.0 fmol mg^{-1} protein, respectively, for rabbits treated with control B_2MM ODN (Fig. 3). This confirms that there is specific attenuation by B_2AS of a single class of receptors of the B_2 type.

These results show that aerosolized A_1AS reached airway smooth muscle; reduced adenosine A_1 receptor number in this tissue in a dose-dependent manner; had no effect on either the adenosine A_2 or bradykinin B_2 receptors; and attenuated the bronchoconstrictor response to adenosine challenge in allergic rabbits. B_2AS provided further evidence of selective attenuation of target gene expression in this system, as it reduced bradykinin B_2 receptor number in airway smooth muscle in a dose-dependent manner, and was without effect on adenosine A_1 or A_2 receptors. Furthermore, all three mismatch control molecules (A_1MM, A_1MM2 and B_2MM), each minimally different from their corresponding antisense molecules, were completely without effect at any receptor at every dose tested. These results provide a clear demonstration of gene-specific antisense effects by aerosolized ODNs in the asthmatic rabbit lung (Table 1).

To assess further the role of the adenosine A_1 receptor in

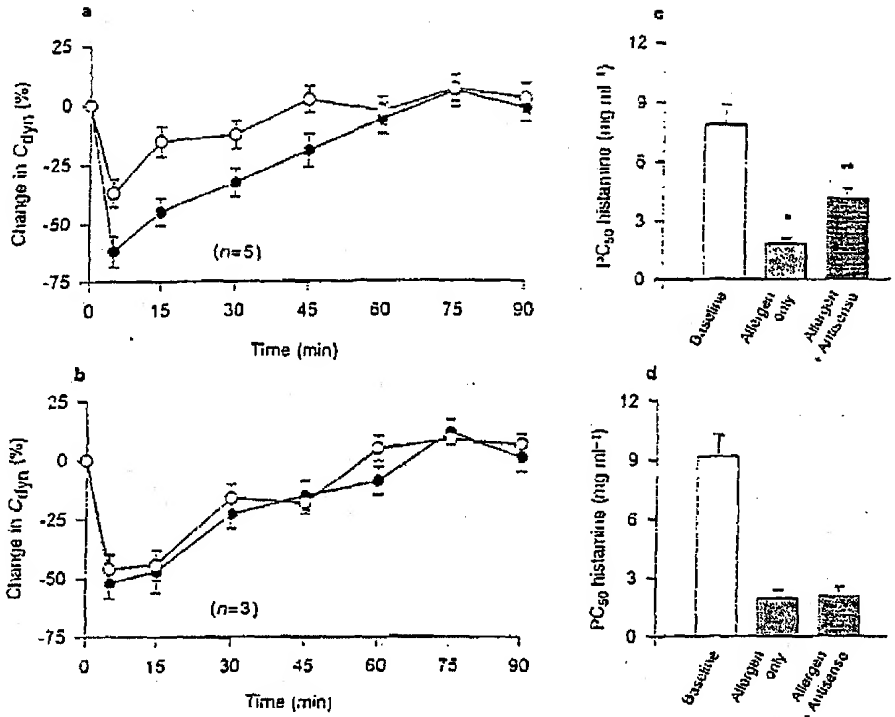


Figure 4 The effect of antisense and mismatch ODNs on allergen-induced airway obstruction and bronchial hyperresponsiveness in allergic rabbits. **a**, Effect of A_1AS antisense ODN on allergen-induced airway obstruction. Allergen only (filled circles), allergen + antisense (open circles). As calculated from the area under the curve, A_1AS significantly inhibited allergen-induced airway obstruction (65%, $P < 0.05$; repeated measures ANOVA and Tukey's t-test. **b**, Lack of effect of mismatch control A_1MM on allergen-induced airway obstruction. Allergen only (filled circles); allergen + antisense (open circles). **c**, Effect of A_1AS antisense ODN on allergen-induced bronchial hyperresponsiveness. As calculated from the PC_{50} histamine, A_1AS significantly inhibited allergen-induced bronchial hyperresponsiveness in allergic rabbits (61%, $P < 0.05$; repeated measures ANOVA and Tukey's t-test). **d**, Lack of effect of A_1MM mismatch control on allergen-induced bronchial hyperresponsiveness. Dynamic compliance (C_{dyn}) is the change in the volume of the lungs divided by the change in the alveolar-distending pressure during the course of a breath.

Table 1 Binding characteristics

Treatment	A_1 receptor		B_2 receptor	
A_1AS (mg)	K_d (nM)	B_{max} (fmol)	K_d (nM)	B_{max} (fmol)
20	0.36 ± 0.023	19 ± 1.52*	0.39 ± 0.031	14.8 ± [illegible]
2	0.38 ± 0.030	32 ± 2.56*	0.41 ± 0.025	15.5 ± [illegible]
0.2	0.37 ± 0.030	49 ± 3.43	0.34 ± 0.024	15.0 ± [illegible]
A_1MM (mg)				
20	0.34 ± 0.027	52.0 ± 3.64	0.35 ± 0.024	14.0 ± [illegible]
2	0.37 ± 0.033	51.8 ± 3.38	0.38 ± 0.028	14.6 ± [illegible]
0.2	0.39 ± 0.027	48.3 ± 2.92	0.40 ± 0.032	15.7 ± [illegible]
B_2AS (mg)				
20	0.36 ± 0.028	45.0 ± 3.15	0.38 ± 0.027	8.7 ± 0.52*
2	0.39 ± 0.035	44.3 ± 2.90	0.34 ± 0.024	11.9 ± 0.75**
0.2	0.40 ± 0.028	47.0 ± 3.76	0.35 ± 0.028	15.1 ± 1.05
B_2MM (mg)				
20	0.39 ± 0.031	42.0 ± 2.94	0.41 ± 0.029	14.0 ± 0.98
2	0.41 ± 0.035	40.0 ± 3.20	0.37 ± 0.030	14.8 ± 0.99
0.2	0.37 ± 0.029	43.0 ± 3.14	0.36 ± 0.025	15.1 ± 1.35
Saline control	0.37 ± 0.041	46.0 ± 5.21	0.39 ± 0.047	14.2 ± 1.35

Binding characteristics of the adenosine A_1-selective ligand [^{3}H]CPCPX and the bradykinin B_2-selective ligand [^{3}H]NPC 17731 in membranes isolated from airway smooth muscle of A_1 adenosine receptor and B_2 bradykinin receptor antisense- and mismatch-treated allergic rabbits. Treatment values refer to total ODN administered in four equivalently divided doses over a 48-h period. Significance was determined by repeated-measures ANOVA and Tukey's protected t-test; N = 4–6 for all groups. All assays were performed in triplicate.
* Significantly different from mismatch control- and saline-treated groups, $P < 0.001$.
** Significantly different from mismatch control- and saline-treated groups, $P < 0.05$.

mediating airway obstruction and bronchial hyperresponsiveness, allergic rabbits were administered A_1AS or control A_1MM followed by bronchoprovocation with house dust mite—allergen (*Dermatophagoides farinae*). In the antisense ODN-treated allergic rabbits there was a 55% improvement in dynamic compliance and a 61% reduction in bronchial hyperresponsiveness in response to histamine challenge (Fig. 4).

These findings suggest that adenosine is an important mediator of both airway obstruction and inflammation, and that some portion of these effects are mediated through the pulmonary adenosine A_1 receptor in the asthmatic lung. They further indicate that the lung may have great potential as a target for antisense ODN-based disease intervention in asthma and related lung pathologies. □

Methods

Preparation of allergic rabbits. Neonatal New Zealand white Pasturella-free rabbit littermates were immunized intraperitoneally within 24 h of birth with 312 antigen units per 0.5 ml house dust mite (*D. farinae*) extract (Berkeley Biologicals) mixed with 10% kaolin[23,24]. Immunizations were repeated weekly for the first month and then every 2 weeks for the next 3 months. At 4 months of age, sensitized rabbits were prepared for aerosol administration[25].

Synthesis and design of antisense ODNs. Phosphorothioate ODNs were synthesized on an Applied Biosystems model 396 oligonucleotide synthesizer using tetraethylthiuram in acetonitrile as sulphurizing agent. Crude ODNs (trityl on) were purified using NENSORB chromatography (DuPont). The

sequence of A1AS was: 5′-GATGGAGGGCGGCATGGCGGG-3′. Two different mismatched ODNs were used as controls and had the sequences: A_1MM 5′-GTAGGTGGCGGGCAAGGCGGG-3′, and A_1MM2 5′-GATGGAGGCGGG-CATGGCGGG-3′. Sequence of B_2AS: 5′-GGTGATGTTGAGCATTTCGGC-3′; sequence of B_2MM: 5′-GGTGAT TTGAGGATTTCGGC-3′.

Administration of aerosolized antisense ODNs and assessment of pulmonary function. Aerosols of either adenosine (0–20 mg ml^{-1}) or antisense or mismatch ODNs (5 mg ml^{-1}) were generated by an ultrasonic nebulizer (Model 646, DeVilbiliss, Somerset, PA), producing aerosol droplets of which 80% were less than 5 μm in diameter. Aerosols were administered directly to the lungs through an intratracheal tube. Rabbits were selected at random, and on day 1 pretreatment values for PC_{50} were obtained for aerosolized adenosine challenge. Animals were subsequently administered aerosolized antisense or mismatch ODN through the intratracheal tube (5 mg in a volume of 1.0 ml), for 2 min, twice daily for 2 days (total dose, 20 mg). On the morning of the third day, post-treatment PC_{50} values were recorded (post-treatment challenge). For Fig. 1, $N = 7$ for mismatch control A_1MM; $N = 4$ for mismatch control A_1MM2; and $N = 8$ for A_1AS antisense ODN. A_1MM2 ODN-treated animals ($N = 4$) were analysed separately and were not part of the crossover experiment. In 6 of the 8 animals treated with antisense ODN and reported in Fig. 1, a PC_{50} value for adenosine could not be obtained up to the limit of solubility of adenosine, 20 mg ml^{-1}. For the purpose of calculation, PC_{50} values for these animals were set at 20 mg ml^{-1}. The values given therefore represent a minimum figure for antisense effectiveness; actual effectiveness was higher. Other groups of allergic rabbits ($N = 4$–6 for each group) were administered doses of 0.5 or 0.05 mg A_1AS or A_1MM in the manner and according to the schedule described above (total doses of 2.0 or 0.2 mg). A_1AS reduced sensitivity to applied adenosine in a dose-dependent manner over the dose range of 0.2 mg total dose (PC_{50} adenosine, 8.32 ± 7.2 mg), 2.0 mg total dose (PC_{50} adenosine 14.0 ± 2.7 mg), and 20 mg total dose (PC_{50} adenosine, 19.5 ± 0.34 mg). No change in PC_{50} adenosine values occurred in rabbits treated with A_1MM control ODN over the same dose range (PC_{50} adenosine, 2.51 ± 0.46 mg at 0.2 mg A_1MM; 3.13 ± 0.71 mg at 2.0 mg A_1MM; and 3.25 ± 0.34 mg at 20 mg A_1MM). Assessment of bronchial hyperresponsiveness using histamine aerosol (Fig. 4) was performed as previously described[25].

Receptor binding. Airway smooth-muscle tissue from tertiary bronchi of rabbits ($N = 4$–6 per group) administered 0.2, 2.0 or 20 mg A_1AS, A_1MM, B_2AS or B_2MM in four divided doses over 48 h was assessed for receptor content[12,26,27]. Protein content was determined as described[28]. No significant inter- or intra-group difference in adenosine A_2 receptor-specific (^{3}H)CGS-21680 binding was observed in airway smooth-muscle plasma membranes isolated from A_1AS-treated animals (specific binding of 2,125 ± 371 c.p.m. per mg protein at 0.2 mg A_1AS; 1,925 ± 370 c.p.m. per mg protein at 0.2 mg A_1AS; and 1,861 ± 281 c.p.m. per mg protein at 0.2 mg A_1AS); from A_1MM-treated animals (specific binding of 2,210 ± 395 c.p.m. per mg protein at 0.2 mg A_1MM; 2,010 ± 390 c.p.m. per mg protein at 0.2 mg A_1MM; and 1,731 ± 276 c.p.m. per mg protein at 0.2 mg A_1MM); from B_2AS-treated animals (specific binding of 2,015 ± 225 c.p.m. per mg protein at 0.2 mg B_2AS; 1,910 ± 342 c.p.m. per mg protein at 0.2 mg B_2AS; and 1,776 ± 349 c.p.m. per mg protein at 0.2 mg B_2AS); or from B_2MM-treated animals (specific binding of 1,914 ± 192 c.p.m. per mg protein at 0.2 mg B_2MM; 1,875 ± 316 c.p.m. per mg protein at 0.2 mg B_2MM; and 1,805 ± 327 c.p.m. per mg protein at 0.2 mg B_2MM). Statistical significance was assessed by repeated measures analysis of variance (ANOVA), and Tukey's t-test.

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